TITLE OF THE INVENTION

DNA Vaccines Against Hantavirus Infections

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INTRODUCTION

Currently, there are four known hantaviruses associated with hemorrhagic fever with renal syndrome (HFRS): Hantaan virus (HTNV), Dobrava-Belgrade virus (DOBV), Puumala virus (PUUV), and Seoul virus (SEOV). Because distinct hantaviruses are usually carried by only one principal rodent host species, their distribution is generally limited to the range of that host (reviewed in Schmaljohn and Hjelle, 1997, *Emerg. Infect. Dis.* 3, 95-104). HTNV, carried by *Apodemus agrarius*, is found in Asia; DOBV, carried by *Apodemus flavicollis*, and PUUV, carried by *Clethrionomys glareolus*, are found in Europe. SEOV is more widely disseminated than any other recognized hantavirus because its host, the common urban rat (*Rattus norvegicus*), is found throughout the world.

Viruses in the *Hantavirus* genus (family *Bunyaviridae*) are enveloped and contain a genome comprised of three single-stranded RNA segments designated large (L), medium (M), and small (S) based on size (reviewed in Schmaljohn, 1996, In The Bunyaviridae Ed. R. M. Elliott. New York, Plenum Press p. 63-90). The hantavirus L segment encodes the RNA dependent RNA polymerase, M encodes two envelope glycoproteins (G1 and G2), and S encodes the nucleocapsid protein (N).

A number of inactivated HFRS vaccines derived from cell culture or rodent brain were developed and tested in Asia (Lee *et al.*, 1990, *Arch. Virol.*, Suppl. 1, 35-47; Song *et al.*, 1992, *Vaccine* 10, 214-216; Lu *et al.*, 1996, *J. Med. Virol.* 49, 333-335). Other vaccine approaches involving recombinant DNA technology include: vaccinia-vectored vaccines (Schmaljohn *et al.* 1990, *J. Virol.* 64, 3162-3170; Schmaljohn *et al.* 1992, *Vaccine* 10, 10-13; Xu *et al.* 1992, *Am. J. Trop. Med. Hyg.* 47, 397-404), protein subunit vaccines expressed in bacteria or insect cells (Schmaljohn *et al.* 1990, supra; Yoshimatsu *et al.*, 1993, *Arch. Virol.* 130, 365-376; Lundkvist *et al.*, 1996, *Virology* 216, 397-406), and a hepatitis core antigen-based recombinant vaccine (Ulrich *et al.*, 1998, *Vaccine* 16, 272-280).

Hantavirus G1, G2, and N proteins are known to elicit immune responses; however, the relative importance these proteins play in providing protection remains an area of investigation. Vaccination with vaccinia recombinants expressing the M segment of either HTNV or SEOV elicited neutralizing antibodies and protected rodents against infection with both HTNV and SEOV, suggesting that an immune response to G1-G2 alone can confer protection (Schmaljohn *et al.* 1990, supra; Xu *et al.* 1992, supra; Chu *et al.* 1995, *J. Virol.* 69, 6417-6423). Similarly, vaccination with G1-G2 protein expressed in insect cells (baculovirus recombinant virus system) elicited neutralizing antibodies and protected hamsters from infection with HTNV (Schmaljohn *et al.* 1990, supra). In both the vaccinia and baculovirus systems, vaccination with G1-G2 provided more complete protection than G1 or G2 alone (Schmaljohn *et al.* 1990, supra). Neutralizing antibody responses to G1-G2 in the aforementioned

vaccine studies correlated with protection, suggesting that neutralizing antibodies play an important role in preventing hantavirus infection. Passive transfer of neutralizing monoclonal antibodies (MAbs) specific to either G1 or G2 protected hamsters against HTNV infection (Schmaljohn *et al.*, 1990, supra; Arikawa *et al.*, 1992, *J. Gen. Virol.* 70, 615-624), supporting the idea that neutralizing antibodies alone can confer protection. The N protein also plays a role in protecting against hantavirus infection. Vaccination with N expressed in bacteria, insect cells, or as chimeric hepatitis B virus (HBV) core particles protected rodents from hantavirus infection (Schmaljohn *et al.*, 1990, supra; Yoshimatsu *et al.* 1993, supra; Lundkvist *et al.*, 1996, supra; Ulrich *et al.*, 1998, supra). Vaccination with vaccinia recombinants expressing the S segment were less conclusive. A construct expressing the HTNV S segment did not protect hamsters from HTNV infection, possibly due to low N expression levels (Schmaljohn *et al.* 1990, supra); and a construct expressing the S segment of SEOV protected three of four gerbils from SEOV infection (Xu *et al.* 1992, supra).

Recombinant vaccinia virus vaccines and vaccinia-based vaccines present disadvantages including the potential for disseminated infection, especially in immunocompromised individuals, since the vaccines consist of live virus. Also, vaccination with these viruses can result in a lesion (pock) that contains infectious virus. Virus from these lesions can be inadvertently spread to other sites (e.g., eyes) or to other individuals. Other drawbacks of vaccinia-based vaccines include discomfort due to swollen lymphnodes and scarring at the site of inoculation.

DNA vaccination mimicks the de novo antigen production and MHC class I-restricted antigen presentation obtainable with live vaccines, without the risks of pathogenic infection.

5 This vaccine approach is advantageous over subunit vaccine which do not elicit a cytotoxic response necessary to prevent the establishment of infection or disease. Also, this DNA vaccine approach allows delivery to mucosal tissues which may aid in

10 conferring resistance to viral introduction since entry of the virus may be through mucosal tissues.

SUMMARY OF THE INVENTION

In this report, we describe a new recombinant DNA vaccine approach that involves vaccination with naked

15 DNA expressing individual hantavirus genome segment cDNAs. Naked DNA vaccination involves delivery of plasmid DNA constructs with a gene(s) of interest into the tissue of the vaccinee (reviewed in Robinson and Torres, 1997, *Semin. Immunol.* 9, 271-283; and

20 Gregoriadis, 1998, *Pharm. Res.* 15, 661-670). The gene(s) of interest, in our case, a hantavirus genome segment, is controlled by a mammalian or virus promoter (e.g., the cytomegalovirus immediate early promoter) that facilitates expression of the naked DNA

25 gene product(s) within the vaccinee's cells. This intracellular expression can elicit both humoral and cell-mediated immune responses (Robinson and Torres, 1997, supra; and Gregoriadis, 1998, supra). Methods of DNA delivery include needle inoculation, oral or

30 pulmonary delivery, and inoculation by particle bombardment (i.e., gene gun). DNA vaccination by each of these methods elicits protective immunity against many different pathogens including numerous viruses (Robinson and Torres, 1997, supra; and Gregoriadis,

35 1998, supra). However, neither an immune response

against hantaviruses nor protection against hantavirus infection have been demonstrated using a DNA vaccine.

In this report, we demonstrate that naked DNA vaccination with the SEOV M or S genome segment

5   elicits SEOV-specific antibody responses in rodents. More importantly, we demonstrate that DNA vaccination with the SEOV M segment elicits neutralizing antibodies and protects hamsters against SEOV infection.

10   Therefore, it is one object of the present invention to provide a hantavirus DNA vaccine comprising a hantavirus genome segment. More specifically, the present invention relates to an SEOV hantavirus DNA vaccine comprising the SEOV M

15   genome segment specified in SEQ ID NO:1 or comprising the SEOV S segment specified in SEQ ID NO:2.

It is another object of the present invention to provide a method for eliciting in a subject an immune response against hantavirus, the method comprising

20   administering to a subject a DNA fragment comprising a genome segment of hantavirus. More specifically, the present invention relates to a method for eliciting an immune response against SEOV hantavirus by providing the M or S genome segment of SEOV.

25   Cross protection against other hantaviruses such as Hantaan virus and Dobrava are also an object of this invention.

In one aspect of the invention, the DNA vaccine is delivered by coating a small carrier particle with

30   the DNA vaccine and delivering the DNA-coated particle into an animal's epidermal tissue via particle bombardment. This method may be adapted for delivery to either epidermal or mucosal tissue, or delivery into peripheral blood cells, and thus may be

used to induce humoral, cell-mediated, and secretory immune reponses in the vaccinated individual.

The DNA vaccine according to the present invention is inherently safe, is not painful to administer, and should not result in adverse side effects to the vaccinated individual. In addition, the invention does not require growth or use of hantavirus, which may be spread by aerosol transmission.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims, and accompanying drawings where:

FIG. 1. SEOV naked DNA expression constructs. The SEOV M or S genome segment was amplified by RT-PCR and cloned into *Not*I and *Bam*HI sites of pWRG7077 (PowderJect Vaccines, Inc., Madison, Wisconsin, described in Schmaljohn et al., 1997, supra). Characteristics of pWRG7077 are similar to those of pWRG1602 described previously (Dimmock, N. J., 1995, *Med. Virol*. 5: 165) and include a human cytomegalovirus early promoter (CMV IE promoter) and intron A, a bovine growth hormone transcription terminator and polyadenylation signal (BGH pA), and a kanamycin resistance gene. CMV IE promoter: cytomegalovirus immediate early promoter and intron A, BGH pA: Bovine growth hormone polyadenylation signal, KAN: kanamycin antibiotic resistance gene.

FIG. 2 A and B. Transient expression from pWRG/SEO-M (SEQ ID NO:3) and pWRG/SEO-S (SEQ ID NO:4). A) IFAT were performed on COS cell monolayers transfected with the SEOV M or S constructs. pWRG/7077-transfected monolayers served as negative

controls. Anti-SEOV polyclonal rabbit antisera was used as the primary antibody. B) COS cells were transfected with the indicated plasmid and radiolabeled expression products were

5 immunoprecipitated with anti-SEOV polyclonal rabbit serum. Molecular size markers (MW) in kDa are shown on the left, and the position of the SEOV G1, G2, and N proteins are shown on the right.

FIG. 3A and 3B. Antibody responses (ELISA) of

10 mice vaccinated with SEOV naked DNA by gene gun or intramuscular needle inoculation. The antibody responses to vaccination with pWRG/SEO-M (panel A) or pWRG/SEO-S (panel B) by gene gun or intramuscular needle inoculation were evaluated by ELISA. Mice

15 vaccinated with negative control plasmid pWRG7077 were evaluated in both ELISAs. Prebleed sera and sera collected 4 weeks after the first vaccination (1 vacc), 4 weeks after the second vaccination (2 vacc), 3 weeks after the final vaccination (3 vacc) were all

20 tested in a single assay. Symbols represent the average values of individual mice serum samples assayed in duplicate.

FIG. 4. Neutralizing antibody responses of mice vaccinated with SEOV naked DNA. PRNT were

25 performed on mice sera collected 3 weeks after the third vaccination by either gene gun or intramuscular needle injection (needle). Bars represent sera from individual mice vaccinated with the indicated immunogen. Sera were heat inactivated (56°C, 30 min)

30 and the assay was performed in the presence of 5% guinea pig complement. PRNT50% titers are expressed as reciprocal of the highest serum dilution resulting in a 50% reduction in plaque number.

FIG. 5. Vaccination with pWRG/SEO-M cross-

35 protects against infection with Hantaan virus. Groups

of 4-5 hamsters were vaccinated with either pWRG/SEO-M
or negative control plasmid (pWRG7077) as described in
Methods below. Three weeks after the final
vaccination, prechallenge serum samples were obtained
and the hamsters were challenged with 1,000 PFU of
Hantaan virus. Twenty-eight days after challenge
postchallenge serum samles were obtained. Pre and
postchallenge serum samples were evaluated by anti-N
ELISA to detect antibody to nucleocapsid, and by a
Hantaan plaque reduction neutralization test (PRNT) to
detect Hantaan neutralizing antibodies. ELISA titers
represent the lowest reciprocal dilution that resulted
in an O.D. value that was greater than the background
O.D. plus three standard deviations. PRNT50% titers
represent the lowest reciprocal dilution that resulted
in a 50% reduction in plaque number in the absence of
serum.

FIG. 6. Vaccination with pWRG/SEO-M cross-
protects against infection with Dobrava virus. Groups
of 5 hamsters were vaccinated with either pWRG/SEO-M
or negative control plasmid (pWRG7077) as described in
Methods. Three weeksafter the final vaccination,
prechallenge serum samples were obtained and the
hamsters were challenged with 1,000 PFU of Dobrava
virus. Twenty-eight days after challenge
postchallenge serum samples were obtained. Pre and
postchallenge serum samples were evaluated by anti-N
ELISA to detect antibody to nucleocapsid, and by a
Dobrava plaque reduction neutralization test (PRNT) to
detect Dobrava neutralizing antibodies. ELISA titers
represent the lowest reciprocal dilution that resulted
in an O.D. value that was greater than the background
O.D. plus three standard deviations. PRNT50% titers
represent the lowest reciprocal dilution that resulted
in a 50% reduction in plaque number in the absence of

serum. The Seoul neutralizing prechallenge antibody titers (elicted by the vaccine) were also measured. The Seoul virus PRNT80% values are shown as empty circles.

5   **FIG. 7.** Vaccination with pWRG/SEO-M fails to cross-protect against infection with Puumala virus. Groups of 5 hamsters were vaccinated with either pWRG/SEO-M or negative control plasmid (pWRG7077) as described in Methods. Three weeks after the final

10 vaccination, prechallenge serum samples were obtained and the hamsters were challenged with 1,000 PFU of Puumala virus. Twenty-eight days after challenge postchallenge serum samples were obtained. Pre and postchallenge serum samples were evaluated by anti-N

15 ELISA to detect antibody to nucleocapsid, and by a Puumala plaque reduction neutralization test (PRNT) to detect Puumala neutralizing antibodies . ELISA titers represent the lowest reciprocal dilution that resulted in an O.D. value that was greater than the background

20 O.D. plus three standard deviations. PRNT50% titers represent the lowest reciprocal dilution that resulted in a 50% reduction in plaque number in the absence of serum. The Seoul neutralizing prechallenge antibody titers (elicted by the vaccine) were also measured.

25 The Seoul virus PRNT80% values are shown as empty circles.

DETAILED DESCRIPTION

In this application is described a composition and method for the vaccination of individuals against

30 hantavirus. The method comprises delivery of a DNA encoding a hantavirus antigen to cells of an individual such that the antigen is expressed in the cell and an immune response is induced in the individual.

DNA vaccination involves administering antigen-encoding polynucleotides *in vivo* to induce the production of a correctly folded antigen(s) within the target cells. The introduction of the DNA vaccine will cause to be expressed within those cells the structural protein determinants associated with the pathogen protein or proteins. The processed structural proteins will be displayed on the cellular surface of the transfected cells in conjunction with the Major Histocompatibility Complex (HMC) antigens of the normal cell. Even when cell-mediated immunity is not the primary means of preventing infection, it is likely important for resolving established infections. Furthermore, the structural proteins released by the expressing transfected cells can also be picked up by antigen-presenting cells to trigger systemic humoral antibody responses.

In order to achieve the immune response sought, a DNA vaccine construct capable of causing transfected cells of the vaccinated individual to express one or more major viral antigenic determinant is necessary. This can be done by identifying regions of the viral genome which code for viral glycoproteins or capsid components, and joining such coding sequences to promoters capable of expressing the sequences in cells of the vaccinee. Alternatively, the viral genome itself, or parts of the genome, can be used.

In one embodiment, the present invention relates to a DNA or cDNA segment which encodes an antigen from a hantavirus. By hantavirus is meant any of the Hemmorhagic fever with renal syndrome (HFRS) hantavirus such as Hantaan virus (HTNV), Dobrava-Belgrade virus (DOBV), Puumala virus (PUUV), and Seoul virus (SEOV), as well as hantavirus pulmonary syndrome (HPS) hantaviruses such as Sin Nombre virus (SNV),

Black Creek Canal virus (BCCV), Bayou virus (BAYV),
New York virus (NYV), Andes virus (ANDV), Laguna Negra
virus (LNV), and any other hantavirus known to cause
disease in humans.

5   More specifically, a hantavirus genome M segment
encoding two envelope glycoproteins (G1 and G2) (SEQ
ID NO:1), and a hantavirus genome S segment encoding
nucleocapsid protein (SEQ ID NO:2) were deduced from
the SEOV, strain SR-11 (Kitamura, T. et al. 1983, *Jpn.*
10   *J. Med. Sci. Biol.* 36, 17-25) viral genome (Arikawa,
J. et al. 1990, *Virology* 176, 114-125). The M segment
(specified in SEQ ID NO:1), deposited in GenBank
accession no. M34882, corresponds to Seoul (SR-11) M
gene from nucleotide 2 to 3602. The S segment (SEQ ID
15   NO:2), having Genbank accession no. M34881,
corresponds to Seoul (SR-11) S gene from nucleotide 2
to 1699.

DNA or polynucleotide sequences to which the
invention also relates include fragments of the M or S
20   gene segment from other Hantaviruses containing
protective epitopes or antigenic determinants. Such
epitopes, which may be conformational, may include
sequences from G1 and/or G2 since monoclonal
antibodies to both G1 and G2 have been shown to
25   neutralize virus and protect rodents in passive
protection experiments. Additionally, the amino
terminus of N is highly immunogenic and others have
shown that other methods of vaccination with the amino
terminal region can confer protection (Lundvist, 1996,
30   supra; Ulrich, 1998, supra).

The derived sequence is not necessarily
physically derived from the nucleotide sequence shown
in SEQ ID NO:1 or SEQ ID NO:2, but may be generated in
any manner, including for example, chemical synthesis
35   or DNA replication or reverse transcription or

transcription, which are based on the information
provided by the sequence bases in the region(s) from
which the polynucleotide is derived. In addition,
combinations of regions corresponding to that of the
5   designated sequence may be modified in ways known in
the art to be consistent with an intended use. The
sequences of the present invention can be used in
diagnostic assays such as hybridization assays and
polymerase chain reaction (PCR) assays for the
10   detection of Hantavirus.

RT-PCR cloning of the M and S genome segments of
SEOV, strain SR-11, was described previously (Arikawa
et al., 1990, Virology 176, 114-125). A DNA fragment
containing Seoul (SR-11) M gene from nucleotide 2 to
15   3602 was excised from YMIS/SR 11-M (Arikawa, 1990,
supra) using Bgl II and Bam HI. This fragment was
ligated into the Bam HI site of pWRG7077 resulting in
pWRG/SEO-M (SEQ ID NO:3). pWRG/SEO-M contains
additional nucleotides derived from cloning vector
20   baculovirus transfer vector YMIS.

A DNA fragment containing Seoul (SR-11) S gene
from nucleotide 2 to 1699 was excised from
pBSKS+/SR11-S (Arikawa, 1990, supra) using EcoRI.
This fragment was subjected to Klenow to blunt the
25   restriction sites and then ligated into Klenow-blunted
Not I site of pWRG7077 (provided by Powderject, Inc.,
Madison, Wisconsin) resulting in pWRG/SEO-S (SEQ ID
NO:4). pWRG/SEO-S contains additional nucleotides
derived from cloning vector pBSKS+.

30   It is understood in the art that certain changes
to the nucleotide sequence employed in a genetic
construct have little or no bearing on the proteins
encoded by the construct, for example due to the
degeneracy of the genetic code. Such changes result
35   either from silent point mutations or point mutations

that encode different amino acids that do not
appreciably alter the behavior of the encoded protein.
It is also understood that portions of the coding
region can be eliminated without affecting the ability

5   of the construct to achieve the desired effect, namely
induction of a protective immune response against
hantavirus. It is further understood in the art that
certain advantageous steps can be taken to increase
the antigenicity of an encoded protein by modifying

10 its amino acid composition. Such changes in amino
acid composition can be introduced by modifying the
genetic sequence encoding the protein. It is
contemplated that all such modifications and
variations of the M and S segments of hantavirus are

15 equivalents within the scope of the present invention.

The DNA encoding the desired antigen can be
introduced into the cell in any suitable form
including, a linearized plasmid, a circular plasmid, a
plasmid capable of replication, an episome, RNA, etc.

20 Preferably, the gene is contained in a plasmid. In a
particularly preferred embodiment, the plasmid is an
expression vector. Individual expression vectors
capable of expressing the genetic material can be
produced using standard recombinant techniques.

25 Please see e.g., Maniatis *et al.*, 1985 Molecular
Cloning: A Laboratory Manual or DNA Cloning, Vol. I
and II (D. N. Glover, ed., 1985) for general cloning
methods.

Therefore, in another embodiment, the present

30 invention relates to a recombinant DNA molecule that
includes a vector and a DNA sequence as described
above. The vector can take the form of a plasmid such
as pCRII (Invitrogen) or pJW4303 (Konishi, E. *et al.*,
1992, *Virology* 188:714), or any expression vector such

as viral vectors e.g. adenovirus or Venezuelan equine encephalitis virus and others known in the art. Preferably, a promoter sequence operable in the target cell is operably linked to the DNA sequence. Several

5   such promoters are known for mammalian systems which may be joined 5', or upstream, of the coding sequence for the encoded protein to be expressed. A suitable promoter is the human cytomegalovirus immediate early promoter. A downstream transcriptional terminator, or

10   polyadenylation sequence, such as the polyA addition sequence of the bovine growth hormone gene, may also be added 3' to the protein coding sequence.

A suitable construct for use in the method of the present invention is pWRG7077 (4326 bp)(PowderJect

15   Vaccines, Inc., Madison, WI), Figure 1. pWRG7077 includes a human cytomegalovirus (hCMV) immediate early promoter (IE) and a bovine growth hormone polyA addition site. Between the promoter and the polyA addition site is Intron A, a sequence that naturally

20   occurs in conjunction with the hCMV IE promoter that has been demonstrated to increase transcription when present on an expression plasmid. Downstream from Intron A, and between Intron A and the polyA addition sequence, are unique cloning sites into which the

25   hantavirus M or S DNA can be cloned. Also provided on pWRG7077 is a gene that confers bacterial host-cell resistance to kanamycin. Any of the fragments that encode hantavirus G1 or G2 or nucleocapsid peptides can be cloned into one of the cloning sites in

30   pWRG7077, using methods known to the art.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA construct. The host cell can be prokaryotic such as

35   *Bacillus* or *E. coli*, or eukaryotic such a

Saccharomyces or *Pichia*, or mammalian cells or insect cells. The vector containing the hantavirus sequence is expressed in the bacteria and the expressed product used for diagnostic procedures or as a vaccine.

5   Please see e.g., Maniatis *et al.*, 1985 <u>Molecular Cloning: A Laboratory Manual</u> or <u>DNA Cloning</u>, Vol. I and II (D. N. Glover, ed., 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a highly purified IgG

10   molecule, an adjuvant, a carrier, or an agent for aid in purification of hantavirus proteins or peptides. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an

15   expression system, the transformed or transfected cells can be used as a source of the protein or peptide encoded by the DNA. The DNA can be used as circular or linear, or linearized plasmid as long as the hantavirus sequences are operably linked to a

20   promoter which can be expressed in the transfected cell.

In this application we describe the elicitation of protective immunity to hantaviruses by DNA vaccines. The DNA can be delivered by injection into

25   the tissue of the recipient, oral or pulmonary delivery and inoculation by particle bombardment (i.e., gene gun). Any of these methods can be used to deliver DNA as long as the DNA is expressed and the desired antigen is made in the cell. Two methods are

30   exemplified in this application, both shown to be successful in eliciting a protective immune response in the vaccinee.

To deliver DNA vaccines by particle bombardment, we chose to use the *PowderJect-XR*™ gene gun device

described in WO 95/19799, 27 July 1995. Other
instruments are available and known to people in the
art. This instrument, which delivers DNA-coated gold
beads directly into epidermal cells by high-velocity

5   particle bombardment, was shown to more efficiently
induce both humoral and cell-mediated immune
responses, with smaller quantities of DNA, than
inoculation of the same DNAs by other parenteral
routes (Eisenbraun, M. *et al.*, 1993, *DNA Cell. Biol.*

10 12: 791; Fynan, E. F. *et al.*, 1993, *Proc. Natl. Acad.
Sci. USA* 90: 11478; Haynes, J. R. *et al.*, 1994, *AIDS
Res. Hum. Retroviruses* 10: Suppl. 2:S43; Pertmer, T.
M. *et al.*, 1995, *Vaccine* 13: 1427). Epidermal
inoculation of the DNA candidate vaccines also offers

15 the advantages of gene expression in an
immunologically active tissue that is generally
exfoliated within 15 to 30 days, and which is an
important natural focus of viral replication after
tick-bite (Bos, J. D., 1997, *Clin. Exp. Immunol.* 107

20 Suppl. 1:3; Labuda, M. *et al.*, 1996, *Virology* 219:357;
Rambukkana, A. *et al.*, 1995, *Lab. Invest.* 73:521;
Stingl, G., 1993, *Recent Results Cancer Res.* 128:45).
Candidate vaccines include particles having M genome
segments, including G1 and/or G2, from different HFRS

25 viruses such as Seoul virus, Hantaan virus, Pumuula
virus, and Dobrava virus, as well as Hantavirus
pulmonary syndrome virus such as Sin Nombre virus,
Black Creek Canal virus, Bayou virus, New York virus,
Andes virus, and Laguna Negra virus. The DNA

30 segments from different viruses can be on different
particles or on the same particle, whichever results
in the desired immune response. In addition, the
present invention relates to a vaccine comprising one
or more DNAs from different hantaviruses. Such a

35 vaccine is referred to as a multivalent vaccine. The

vaccine is designed to protect against pathologies resulting from exposure to one or several hantaviruses. The vaccine can also be combined with reagents which increase the antigenicity of the

5 vaccine, or reduce its side effects.

The technique of accelerated particles gene delivery or particle bombardment is based on the coating of DNA to be delivered into cells onto extremely small carrier particles, which are designed

10 to be small in relation to the cells sought to be transformed by the process. The DNA sequence containing the desired gene can be simply dried onto a small inert particle. The particle may be made of any inert material such as an inert metal (gold, silver,

15 platinum, tungsten, etc.) or inert plastic (polystyrene, polypropylene, polycarbonate, etc.). Preferably, the particle is made of gold, platinum or tungsten. Most preferably, the particle is made of gold. Suitably, the particle is spherical and has a

20 diameter of 0.5 to 5 microns, preferably 1 to 3 microns.

The DNA sequence containing the desired gene prepared in the form suitable for gene introduction can be simply dried onto naked gold or tungsten

25 pellets. However, DNA molecules in such a form may have a relatively short period of stability and may tend to degrade rather rapidly due to chemical reactions with the metallic or oxide substrate of the particle itself. Thus, if the carrier particles are

30 first coated with an encapsulating agent, the DNA strands have greatly improved stability and do not degrade significantly even over a time period of several weeks. A suitable encapsulating agent is polylysine (molecular weight 200,000) which can be

35 applied to the carrier particles before the DNA

molecules are applied. Other encapsulating agents,
polymeric or otherwise, may also be useful as similar
encapsulating agents, including spermidine. The
polylysine is applied to the particles by rinsing the
5   gold particles in a solution of 0.02% polylysine and
then air drying or heat drying the particles thus
coated. Once the metallic particles coated with
polylysine were properly dried, DNA strands are then
loaded onto the particles.

10   The DNA is loaded onto the particles at a rate of
between 0.5 and 30 micrograms of DNA per milligram of
gold bead spheres. A preferable ratio of DNA to gold
is 0.5-5.0 ug of DNA per milligram of gold.
A sample procedure begins with gamma irradiated
15   (preferably about 30 kGy) tefzel tubing. The gold is
weighed out into a microfuge tube, spermidine (free
base) at about 0.05 M is added and mixed, and then the
DNA is added. A 10% CaCl solution is incubated along
with the DNA for about 10 minutes to provide a fine
20   calcium precipitate. The precipitate carries the DNA
with it onto the beads. The tubes are microfuged and
the pellet resuspended and washed in 100% ethanol and
the final product resuspeded in 100% ethanol at
0.0025mg/ml PVP. The gold with the DNA is then
25   applied onto the tubing and dried.

The general approach of accelerated particle gene
transfection technology is described in U.S. Patent
No. 4,945,050 to Sanford. An instrument based on an
improved variant of that approach is available
30   commercially from PowderJect Vaccines, Inc., Madison
Wisconsin, and is described in WO 95/19799. All
documents cited herein *supra* and infra are hereby
incorporated in their entirety by reference thereto.
Briefly, the DNA-coated particles are deposited onto
35   the interior surface of plastic tubing which is cut to

a suitable length to form sample cartridges. A sample cartridge is placed in the path of a compressed gas (e.g., helium at a pressure sufficient to dislodge the particles from the cartridge e.g., 350-400 psi). The

5 particles are entrained in the gas stream and are delivered with sufficient force toward the target tissue to enter the cells of the tissue. Further details are available in the published apparatus application.

10 The coated carrier particles are physically accelerated toward the cells to be transformed such that the carrier particles lodge in the interior of the target cells. This technique can be used either with cells in vitro or in vivo. At some frequency,

15 the DNA which has been previously coated onto the carrier particles is expressed in the target cells. This gene expression technique has been demonstrated to work in prokaryotes and eukaryotes, from bacteria and yeasts to higher plants and animals. Thus, the

20 accelerated particle method provides a convenient methodology for delivering genes into the cells of a wide variety of tissue types, and offers the capability of delivering those genes to cells in situ and in vivo without any adverse impact or effect on

25 the treated individual. Therefore, the accelerated particle method is also preferred in that it allows a DNA vaccine capable of eliciting an immune response to be directed both to a particular tissue, and to a particular cell layer in a tissue, by varying the

30 delivery site and the force with which the particles are accelerated, respectively. This technique is thus particularly suited for delivery of genes for antigenic proteins into the epidermis.

A DNA vaccine can be delivered in a non-invasive

35 manner to a variety of susceptible tissue types in

order to achieve the desired antigenic response in the
individual. Most advantageously, the genetic vaccine
can be introduced into the epidermis. Such delivery,
it has been found, will produce a systemic humoral
5   immune response.

To obtain additional effectiveness from this
technique, it may also be desirable that the genes be
delivered to a mucosal tissue surface, in order to
ensure that mucosal, humoral and cellular immune
10   responses are produced in the vaccinated individual.
There are a variety of suitable delivery sites
available including any number of sites on the
epidermis, peripheral blood cells, i.e. lymphocytes,
which could be treated in vitro and placed back into
15   the individual, and a variety of oral, upper
respiratory, and genital mucosal surfaces.

Gene gun-based DNA immunization achieves direct,
intracellular delivery of DNA, elicits higher levels
of protective immunity, and requires approximately
20   three orders of magnitude less DNA than methods
employing standard inoculation.

Moreover, gene gun delivery allows for precise
control over the level and form of antigen production
in a given epidermal site because intracellular DNA
25   delivery can be controlled by systematically varying
the number of particles delivered and the amount of
DNA per particle. This precise control over the level
and form of antigen production may allow for control
over the nature of the resultant immune response.
30   The term transfected is used herein to refer to
cells which have incorporated the delivered foreign
DNA vaccine, whichever delivery technique is used.

It is herein disclosed that when inducing
cellular, humoral, and protective immune repsonses
35   after DNA vaccination the preferred target cells are

epidermal cells, rather than cells of deeper skin layers such as the dermis. Epidermal cells are preferred recipients of DNA vaccines because they are the most accessible cells of the body and may,

5 therefore, be immunized non-invasively. Secondly, in addition to eliciting a humoral immune response, DNA immunized epidermal cells also elicit a cytotoxic immune response that is stronger than that generated in sub-epidermal cells. Delivery to epidermis also

10 has the advantages of being less invasive and delivering to cells which are ultimately sloughed by the body.

 Although it can be desirable to induce an immune response by delivering genetic material to a target

15 animal, merely demonstrating an immune response is not necessarily sufficient to confer protective advantage on the animal. What is important is to achieve a protective immune response that manifests itself in a clinical difference. That is, a method is effective

20 only if it reduces the severity of the disease symptoms. It is preferred that the immunization method be at least 20% effective in preventing death in an immunized population after challenge with hantavirus. More preferably, the vaccination method

25 is 50% or more effective, and most preferably 70-100% effective, in preventing death in an immunized population. The vaccination method is shown herein to be 100% effective in the hamster model for hantavirus. Hamsters have been used extensively as the laboratory

30 model of choice for assessment of protective immune responses to hantaviruses. In contrast, unimmunized animals are uniformly infected by challenge with hantavirus. Our results indicate that vaccination with SEOV M genome segment protects against Hantaan

35 virus (HTNV), and against Dobrava virus (DBOV).

Generally, the DNA vaccine administered may be in an amount of about 1-5 ug of DNA per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine for eliciting an immune response against one or more viruses, may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

In another embodiment, the present invention provides reagents useful for carrying out the present process. Such reagents comprise a DNA fragment containing M or S or both gene segments from hantavirus, and a small, inert, dense particle. The DNA fragment, and dense particle are those described above.

Preferably, the DNA is frozen or lyophilized, and the small, inert, dense particle is in dry powder. If a coating solution is used, the dry ingredients for the coating solution may be premixed and premeasured

and contained in a container such as a vial or sealed envelope.

The present invention also provides kits which are useful for carrying out the present invention.

5 The present kits comprise a first container means containing the above-described frozen or lyophilized DNA. The kit also comprises a second container means which contains the coating solution or the premixed, premeasured dry components of the coating solution.

10 The kit also comprises a third container means which contains the small, inert, dense particles in dry powder form or suspended in 100% ethanol. These container means can be made of glass, plastic or foil and can be a vial, bottle, pouch, tube, bag, etc. The

15 kit may also contain written information, such as procedures for carrying out the present invention or analytical information, such as the amount of reagent (e.g. moles or mass of DNA) contained in the first container means. The written information may be on

20 any of the first, second, and/or third container means, and/or a separate sheet included, along with the first, second, and third container means, in a fourth container means. The fourth container means may be, e.g. a box or a bag, and may contain the

25 first, second, and third container means.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to

30 be limiting thereof.

The following materials and method were used in the examples below.

MATERIALS AND METHODS

Viruses, cells, and medium

5   SEOV, strain SR-11 (Kitamura et al., 1983, Jpn. J. Med. Sci. Biol. 36, 17-25) was propagated in Vero E6 cells (Vero C1008; ATCC CRL 1586). Transient expression experiments were performed with COS cells (COS-7; ATTC CRL1651). Both cell types were

10   maintained in Eagle's minimal essential medium with Earle's salts (EMEM) containing 10% fetal bovine serum, 10 mM HEPEs pH 7.4; and antibiotics (penicillin [100 U/ml], streptomycin [100 µg/ml], and gentamycin [50 µg/ml])(cEMEM).

15   **Construction of SEOV M and S naked DNA plasmids**

RT-PCR cloning of the M and S genome segments of SEOV, strain SR-11, was described previously (Arikawa et al., 1990, Virology 176, 114-125). The cDNA representing each genome segment was subcloned into

20   the Not I or Bam HI site of a previously described naked DNA vector pWRG7077 (Schmaljohn et al., 1997, J. Virol. 64, 3162-3170). Plasmid DNA was purified using Qiagen maxiprep DNA purification kits according to the manufacturer's directions (Cat. no. 12163, Qiagen).

25   The DNA we used for both intramuscular needle inoculation and epidermal gene gun inoculation was not prepared using endo-free qiagen DNA purification kits, and therefore, the DNA was not endotoxin-free (Qiagen plasmid kits yield ~9.3 endotoxin units per µg DNA

30   [QIAGEN Plasmid Purification Handbook 01.97]). To control for possible immunostimulatory effects of endotoxin, our negative control plasmid DNA was prepared in precisely the same was as the vaccine candidates.

Indirect fluorescent antibody test (IFAT)

IFAT was a modification of a previously described procedure (Kamrud *et al.*, 1995, *Exp. Parasitol.* 81, 394-403). COS cells grown on 15mm glass cover slips in 12-well cell culture plates were transfected with 1 µg of plasmid DNA using Fugene6 (Boehringer Mannheim) as described by the manufacturer. Twenty-four hr posttransfection, cover slips were rinsed once with PBS (pH 7.4), fixed with acetone for 10 min at room temperature, and then reacted with anti-SEOV polyclonal antiserum (rabbit), or sera from vaccinated animals. Antibodies were diluted in PBS containing 3% fetal bovine serum (FBS) and then incubated on transfected cells for 30 min at 37°C. Cover slips were rinsed three times with PBS and incubated for 30 min at 37°C with biotinylated donkey anti-rabbit followed by streptavidin conjugated fluorescein (Amersham), or fluorescein-labeled goat anti-hamster antibody (Kirkegaard & Perry Laboratories) diluted in PBS,3% FBS. Evans blue (Fisher) was included in the secondary antibody solution as a counter stain. Cover slips were rinsed three times with PBS and once with deionized water and then were placed on a drop of fluorescence mounting medium (DAKO) on glass slides. Cells were observed with a Zeiss Axioplan fluorescence microscope.

Immunoprecipitation

COS cells grown in T-25 cell culture flasks were transfected with 8 µg of plasmid DNA using Fugene6 (Boehringer Mannheim). After 24 hr, expression products were radiolabeled with Promix ($[^{35}S]$-methionine and $[^{35}S]$-cysteine, Amersham) and immunoprecipitated as described previously (Schmaljohn *et al.*, 1997, supra).

Vaccination with the gene gun

Cartridges for the gene gun were prepared as described previously (Eisenbraun *et al.*, 1993, *DNA Cell. Biol.* 12, 791-797; Schmaljohn *et al.* 1997,

5   supra). Briefly, ~1.5 µg of DNA was precipitated onto 0.5 mg of ~ 2 µm diameter gold beads (Degussa). DNA-coated gold beads were used to coat the inner surface of Tefzel tubing (McMaster-Carr). Cartridges were made by cutting the DNA-gold-coated Tefzel tubing into

10   ~0.5-inch sections, each containing ~0.5 mg of gold, coated with ~0.75 µg of DNA (~50% of the precipitated DNA bound to the gold as determined by elution and fluorometric assay). To vaccinate animals, abdominal fur was removed with clippers and DNA-coated gold was

15   administered to nonoverlapping sites on the abdominal epidermis by using the gene gun (Powderject-XR delivery device, Powderject Vaccines, Inc.) as described previously (Pertmer *et al.*, 1995, *Vaccine* 13, 1427-1430). BALB/c mice (National Cancer

20   Institute) and outbred Syrian hamsters (Charles River) were vaccinated with two or four cartridges, respectively, under 400 p.s.i. of helium pressure. This research was conducted in accordance with procedures described in the Guide for the Care and Use

25   of Laboratory Animals (National Institute of Health, 1996). The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Vaccination by intramuscular needle inoculation

30   Plasmid DNA (25 µg) suspended in 50 µl of PBS (pH 7.4) was injected into the gastrocnemius muscle of anesthetized mice with a 28.5-gauge needle.

ELISA

To detect SEOV G1 and G2, ELISA plates (Costar)

35   were coated with SEOV-infected Vero E6 cell lysates

[⁻Co-irradiated (3 million rads) to inactivate virus]
diluted in carbonate buffer (pH. 9.6) overnight at
4°C. Plates were blocked with PBS, 3% skim milk,
0.05% Tween-20 (blocking solution) for 1 hr at 37°C

5 and then washed once with PBS, 0.05% Tween-20 (wash
solution). Mouse or hamster sera diluted in blocking
solution were added to the wells, and plates were
incubated as before. Plates were washed four times
with wash solution and then incubated for 1 hr with

10 horseradish peroxidase (HRP)-conjugated goat anti-
mouse IgG (Cat. no. A-4416, Sigma) at a dilution of
1/1,000 or peroxidase-labeled goat anti-hamster
antibody (Cat. no. 14-22-06, Kirkegaard & Perry
Laboratories) diluted 1/2,000 in blocking solution.

15 Plates were washed as before and then incubated for 10
min at room temperature with tetra-methylbenzidine
substrate (TMB, Cat. no. 50-76-04, Kirkegaard & Perry
Laboratories). The colorimetric reaction was stopped
by adding Stop solution (Cat. no. 50-85-04, Kirkegaard

20 & Perry Laboratories) and the optical density (O.D.),
450 nm, was determined. The method for detecting SEOV
N-specific antibodies was adapted from a previously
described technique (Elgh et al., 1997, J. Clin.
Microbiol. 35, 1122-1130). Amino acids 1-117 of the

25 nucleocapsid protein of SEOV (strain SR-11) were
expressed as a histidine-tagged fusion protein by
using the pRSET plasmid (Invitrogen) in Escherichia
coli BL21 (DE3) (Novagen, Inc.) and purified by
affinity chromatography on Ni-NTA columns (Qiagen).

30 Antigen was diluted in carbonate buffer (pH 9.6),
added to 96-well microtiter plates (Maxisorp; NUNC)
(0⁻.2 μg in 100 μl per well), and incubated overnight
at 4°C. Plates were washed in deionized water and
incubated with blocking solution for 30 min at room

35 temperature. After plates were washed in deionized

water, 100 µl of serum (diluted in blocking buffer
containing *E. coli* antigen extract [0.6 mg/ml]) was
added to duplicate wells. Plates were incubated at
37°C for 1 h and again washed as before. Addition of
5   HRP secondary antibody and colorimetric detection were
added as described above for the SEOV G1-G2 ELISA.
End-point titers were determined as the highest
dilution with an O.D. greater than the mean O.D. value
of serum samples from negative control plasmid
10   (pWRG7077)-vaccinated animals plus three standard
deviations.

Plaque reduction neutralization tests (PRNT)

Neutralization assays were performed essentially
as previously described (Chu *et al.*, 1995, *J. Virol.*
15   69, 6417-6423). Serum samples were diluted in cEMEM
and then combined with an equal volume (111 µl) of
cEMEM containing ~75 PFU of SEOV. This serum-virus
mixture was incubated overnight at 4°C and then 200
µl/well was added to 6well plates containing Vero E6
20   monolayers 3-7 days old. After a 1-hr adsorption at
37°C, 3 ml of overlay medium (Earl's basal minimal
essential medium (EBME), 10 mM HEPEs, 0.6% agarose
[Sea Kem ME agarose], 8 mM L-glutamine, antibiotics)
containing 10% FBS and 1x nonessential amino acid
25   mixture (GIBCO BRL) was added to each well. Plates
were incubated at 37°C for 7 days and then stained by
adding 2 ml/well of overlay medium containing 5% FBS
and 5% neutral red solution (GIBCO BRL). Plaques were
counted after 2 days at 37°C. Serum samples were heat
30   inactivated (56°C, 30 min) before assay. In
experiments where complement was used, serum and virus
were incubated overnight in the presence of 5% guinea
pig complement (Cat. no. ACL-4051, Accurate Chemical
and Scientific Corporation).

Challenge with SEOV

Syrian hamsters were injected intramuscularly (caudal thigh) with a 25-gauge needle with 10^3 PFU of SEOV diluted in 0.2 ml sterile PBS (pH 7.4). The 10^3

5   PFU dose was based on previously pulished work that reported 100% infection of hamsters after this challenge dose and route (Schmaljohn *et al.*, 1990, supra; Chu *et al.*, 1995, supra). Twenty-eight days after challenge, the hamsters were anesthetized and

10 exsanguinated by cardiac puncture. Postchallenge serum was evaluated for the presence of anti-SEOV antibody by IFAT, ELISA, and PRNT. A dramatic rise in postchallenge antibody titer to viral proteins other than the immunogens indicated that the hamster was

15 infected with SEOV.

Cross protection assay. Groups of 4-5 hamsters were vaccinated with either pWRG/SEO M or negative control plasmid (pWRG7077). Vaccinations consisted of four gene gun administrations (~1.5 ug DNA per

20 administration) three times at three week intervals. Three weeks after the final vaccination, prechallenge serum samples were obtained and the hamsters were challenged with 1,000 PFU of Hantaan virus, 1,000 PFU of Dobrava virus, or 1,000-2,000 PFU of Puumala virus.

25 Twenty-eight days after challenge postchallenge serum samples were obtained. Pre and postchallenge serum samples were evaluated by anti-N ELISA to detect antibody to nucleocapsid, by a Hantaan, Dobrava, or Puumula plaque reduction neutralization test (PRNT) to

30 detect the resective neutralizing antibodies depending on the challenge virus. Details of vaccination procedures, anti-N ELISA, and PRNT are as described in Hooper et al, Virology, March 15,1999.

EXAMPLE 1

Transient expression of hantavirus genes.

The cDNA representing the M (SEQ ID NO:1) or S (SEQ ID NO:2) gene segment of the SR-11 strain of SEOV

5 was subcloned into the naked DNA expression vector pWRG7077 downstream of the cytomegalovirus immediate early promoter to yield pWRG/SEO-M (SEQ ID NO:3) and pWRG/SEO-S (SEQ ID NO:4), respectively (Fig. 1). To assess expression by the M and S constructs, these

10 plasmids were transfected into COS cells and the expressed proteins were either immunostained or immunoprecipitated. The presence of stained cells in transfected monolayers incubated with anti-SEOV polyclonal sera indicated that SEOV reactive

15 protein(s) were expressed from both pWRG/SEO-M and pWRG/SEO-S (Fig. 2A). Radiolabeled expression products were immunoprecipitated with anti-SEOV polyclonal antisera and analyzed by SDS-PAGE (Fig. 2B). Immunoprecipitated proteins had apparent

20 molecular sizes expected for G1, G2, and N (80 kDa, 56 kDa, and 48 kDa)(Elliot *et al.* 1984, *J. Gen. Virol.* 65, 1285-1293; Schmaljohn *et al.* 1986, *Virology* 155, 633-643). Additional protein bands with apparent molecular masses of ~37 kDa and ~28 kDa were co-

25 precipitated with N. These polypeptides were immunoprecipitated by a N amino-terminus-specific monoclonal antibody, and could be detected by Western blot, suggesting they represent truncated forms of N (data not shown).

30 ## EXAMPLE 2

Vaccination of mice with pWRG/SEO-M or pWRG/SEO-S. To determine if either the M or S construct could elicit an antibody response in mice, groups of 10 6- to 8-week-old female BALB/c mice were

35 vaccinated with pWRG/SEO-M, pWRG/SEO-S, or negative

control DNA (pWRG7077) by two different routes: inoculation of the epidermis by gene gun, or inoculation of the gastrocnemius muscle by needle injection. Mice received a priming vaccination

5 followed by two boosts at 4 week intervals. Blood samples collected before each vaccination and 3 weeks after the final boost were screened for a SEOV-specific antibody response by ELISA. Both the M and S constructs elicited a SEOV-specific antibody response

10 by both routes of DNA administration (Fig. 3). Most mice vaccinated with SEOV S by gene gun exhibited specific antibodies after only one vaccination, and the antibody responses increased after subsequent vaccinations (Fig. 3B). To compare the relative

15 antibody responses, end-point ELISA titers were determined for the final blood samples (Table 1). Gene gun vaccination resulted in higher seroconversion frequencies and geometric mean titers (GMT) than intramuscular vaccination with either construct.

20 Table 1: ELISA Titers in Mice Vaccinated with SEOV Naked DNA Vaccines

Inoculated DNA	Vaccine route[a]	Positive/ total	End point titer range	GMT[b]
pWRG/SEO-M	g.g.	9/10	200-1600	303
pWRG/SEO-M	i.m.	7/10	100-400	132
pWRG/SEO-S	g.g.	10/10	6400-12800	7352
pWRG/SEO-S	i.m.	9/10	800-6400	1213

Note. Mice were vaccinated three times at 4-week intervals.

30 Titers were determined for sera collected three weeks after the final boost.

[a]g.g. indicates gene gun; i.m., intramuscular needle inoculation.

[b]Geometric mean titer of all animals in group.

To determine if naked DNA vaccination elicited neutralizing antibodies, we performed plaque reduction neutralization tests (PRNT) on sera collected 3 weeks after the final boost. All (10/10) mice that were vaccinated with pWRG/SEO-M by gene gun exhibited PRNT50% titers ranging from 40 to 1280 (Fig. 4). Only 1/10 mice vaccinated with pWRG/SEO-M by needle injection exhibited a detectable neutralizing antibody response (PRNT50% = 40). Mice vaccinated with pWRG/SEO-S or pWRG7077 by either gene gun or needle inoculation did not develop neutralizing antibodies (Fig. 4).

EXAMPLE 3

Vaccination and challenge of hamsters. To study the protective efficacy of the naked DNA hantavirus vaccines, groups of five hamsters were vaccinated with either pWRG/SEO-M, pWRG/SEO-S, or negative control plasmid pWRG7077 by gene gun (a priming vaccination followed by two boosts at 4-week intervals) and then challenged with SEOV 6 weeks after the final boost. IFAT, ELISA, and PRNT were performed on serum samples taken before vaccination, on the day of challenge, and 4 weeks after challenge. Because there are no disease models for hantavirus in adult rodents, we evaluated whether or not our DNA vaccines could protect against infection rather than prevent disease.

IFAT and ELISA results demonstrated that before vaccination, none of the hamsters was positive for either SEOV G1-G2 or N protein (data not shown). After vaccination (prechallenge sera drawn on day of challenge), five of five hamsters vaccinated with pWRG/SEO-M developed anti-G1-G2 antibodies, and four of five hamsters vaccinated with the pWRG/SEO-S exhibited anti-N antibodies by IFAT (Table 2). The

prechallenge ELISA results detected similar antibody
responses except that only four of five pWRG/SEO-M
vaccinated hamsters were positive in the anti-SEOV
ELISA. The pWRG7077 vaccinated hamsters failed to

5   develop both G1-G2- and N-specific antibodies by IFAT
and ELISA before challenge.

After challenge, serum samples from hamsters
previously vaccinated with pWRG/SEO-M remained
negative for anti-nucleocapsid antibody by IFAT

10   indicating the animals were not infected with SEOV.
In contrast, postchallenge serum samples from
pWRG/SEO-S and pWRG7077 vaccinated hamsters were
positive for both G1-G2 and N antibodies, indicating
that the animals were infected when challenged with

15   SEOV. Postchallenge ELISA results were similar to the
IFAT results; however, three of five of the pWRG/SEO-M
vaccinated hamsters exhibited a weak anti-N response
after challenge indicating that, although the IFAT
were negative, the hamsters had developed antibodies

20   to the challenge virus.

Before vaccination, none of the hamsters had SEOV
neutralizing antibody titers (data not shown). After
vaccination, all hamsters vaccinated with pWRG/SEO-M
developed neutralizing antibodies with PRNT80% titers

25   ranging from 160 to 640 (Table 2). Neutralizing
antibody was not detected in pWRG/SEO-S and pWRG7077
prechallenge sera from vaccinated hamsters. After
SEOV challenge, neutralizing antibody titers of the
pWRG/SEO-M immunized animals remained essentially the

30   same as before challenge (plus or minus twofold). In
contrast, the pWRG7077 and pWRG/SEO-S postchallenge
neutralizing antibody titers increased significantly
from <20 to between 1,280 and >81,920.

Table 2. Serological responses and protection of hamsters vaccinated with naked DNA vaccines.

Vaccine[a]	Hamster	IFAT[b] anti-N		anti-G1-G2		PRNT$_{50,c}$		Protection
		pre	post	pre	post	pre	post	
pWRG/SEO-M	1	-	-	+	+	320(20)	640	yes
	2	-	-	+	+	320(40)	640	yes
	3	-	-	+	+	320(20)	1280	yes
	4	-	-	+	+	80(<20)	160	yes
	5	-	-	+	+	640(20)	1280	yes
PWRG/SEO-S	1	+	+	-	+	<20(<20)	>640	no
	2	+	+	-	+	<20(<20)	>640	no
	3	+	+	-	+	<20(<20)	>640	no
	4	+	+	-	+	<20(<20)	>640	no
	5	-	+	-	+	<20(<20)	>640	no
PWRG7077	1	-	+	-	+	<20(<20)	>640	no
	2	-	+	-	+	<20(<20)	>640	no
	3	-	+	-	+	<20(<20)	>640	no
	4	-	+	-	+	<20(<20)	>640	no
	5	-	+	-	+	<20(<20)	>640	no

[a] Three vaccinations consisting of 4 gene gun cartridges (about 1.5 ug/cartridge) administered at 4 week intervals.

[b] Sera were screened at 1:200 in IFAT sing pWRG/SEO-S or pWRG/SEO-M trasnfected cells as target antigen for anti-n and anti-G1-G2, respectively. - indicates no reactivity; + indicates reactivity.

[c] PRNT values are reciprocal serum dilutions reducing plaque number by 50%. Assays were performed in the presence of 5% guinea pig complement. Numbers in parenthesis are PRNT values of heated serum in the absence of complement. pre, prechallenge serum samples from day of challenge; post, poschallenge serum samples from 4 weeks post challenge.

During the course of this study, we observed that PRNT titers decreased dramatically if sera were heat-inactivated (56°C, 30 min). To test the possibility that the neutralizing activity was complement-

5 enhanced, we performed PRNT assays in the presence of 5% guinea pig complement and found that adding complement increased the PRNT titers of heated serum 4- to 32-fold (Table 2). Complement alone had no effect on SEOV plaque number (data not shown).

10 **Example 4**

Vaccination with pWRG/SEO-M cross-protects against infection with Hantaan virus

Hamsters vaccinated with pWRG/SEO-M had no detectable anti-N antibody prior to challenge, and the

15 postchallenge anti-N response was undetectable or very low (<= 1:200) (Fig. 6). In contrast, hamsters vaccinated with the negative control plasmid exhibited a drammatic rise in anti-N antibody (~2 log increase over background). The PRNT indicated that vaccination

20 with pWRG/SEO-M elicited detectable levels of cross neutralizing antibodies in 3 of 4 hamsters. The PRNT titers of these hamsters did not rise after challenge. In the one hamster with undetectable (<1:20) Hantaan neutralizing antibody, the PRNT titer did rise;

25 however, the increase was at least 32-fold lower than that seen in the hamsters vaccinated with the negative control plasmid.

The low or absent increase in antibody titers to Hantaan structural proteins (as measured by ELISA and

30 PRNT) after challenge with Hantaan virus indicates that vaccination with the Seoul virus M gene (pWRG/SEO-M) confers protection against infection with Hantaan virus.

Example 5

Vaccination with pWRG/SEO-M cross-protects against infection with Dobrava virus.

Hamsters vaccinated with pWRG/SEO-M had no
5   detectable anti-N antibody prior to challenge, and the
postchallenge anti-N response was undetectable. In
contrast, all but one of the hamsters vaccinated with
the negative control plasmid exhibited a drammatic
rise in anti-N antibody (postchallenge anti-N titers
10   of between 1:200 and 1:204,800). The PRNT indicated
that vaccination with pWRG/SEO-M elicited low levels
of cross neutralizing antibodies in 5 of 5 hamsters.
The PRNT titers of these hamsters did not rise above
1:80 after challenge. In contrast, all but one
15   hamster vaccinated with the negative control plasmid
exhibited a high postchallenge PRNT (1:5120)
indicating the hamsters were infected with Dobrava
virus. It remains unclear why animal #182 was not
infected.

20   The low or absent increase in antibody titers to
Dobrava structural proteins (as measured by ELISA and
PRNT) after challenge with Dobrava virus indicates
that vaccination with the Seoul virus M gene
(pWRG/SEO-M) confers protection against infection with
25   Dobrava virus.

Example 5

Vaccination with pWRG/SEO-M fails to cross-protect against infection with Puumala virus.

Hamsters vaccinated with pWRG/SEO-M had no
30   detectable anti-N antibody prior to challenge. After
challenge, all but one hamster had postchallenge anti-
N response greater than or equal to 1:200 indicating
the hamsters were infected with Puumala virus. The
negative control hamsters had similar pre and
35   postchallenge anti-N titers. The PRNT resulsts

indicated that, although vaccination with pWRG/SEO-M elicited neutralizing antibodies against Seoul virus (empty circles), these antibodies did not cross-neutralizing Puumala virus, and failed to protect against infection. The postchallenge Puumala virus PRNT titers of the pWRG/SEO-M vaccinated and negative control vaccinated hamsters were all similar (greater than or equal to 1:160), exept one negative control hamster that was not infected. It remains unclear why negative control animal #220 was not infected.

The increase in antibody titers to Puumala virus structural proteins (as measured by ELISA and PRNT) after challenge with Puumala virus indicates that vaccination with the Seoul virus M gene (pWRG/SEO-M) fails to confers protection against infection with Puumala virus. It should be noted that the hamster with the highest prechallenge anti-Seoul virus PRNT titer was 1:640 (animal #209). It is possible that animals with higher prechallenge neutralizing antibody titers might be cross-protected against Puumala virus.

DISCUSSION

We report that naked DNA shows promise as a way to vaccinate against hantaviruses. Our results demonstrate that vaccination with cDNA representing either the SEOV M or S genome segment elicited antibody responses in BALB/c mice and Syrian hamsters. Hamsters vaccinated with DNA expressing the M segment, but not S segment, developed neutralizing antibodies and were protected against infection with SEOV.

In our initial immunogenicity experiments, gene gun vaccination of BALB/c mice resulted in higher seroconversion rates than intramuscular needle inoculation for both pWRG/SEO-M and pWRG/SEO-S, as measured by ELISA and PRNT. These preliminary data prompted us to focus on the gene gun rather than the

intramuscular route of DNA delivery.   However, because
only one intramuscular DNA dose was tested (25 μg per
animal), it is possible that the seroconversion rate
and/or titers could be increased by adjusting
5   parameters such as the amount of DNA injected, site of
injection, or vaccination schedule.   It is also
possible that needle inoculation elicited a
predominantly cell-mediated rather than humoral
response to the DNA vaccination, as has been seen by
10   others (Feltquate *et al.*, 1997, *J. Immunol.* 158, 2278-
2284; Robinson and Torres, 1997, *Semin. Immunol.* 9,
271-283; Gregoriadis, 1998, *Pharm. Res.* 15, 661-670).
        Gene gun vaccination with pWRG/SEO-M protected
all hamsters against infection with SEOV as measured
15   by 1) undetectable, or barely detectable,
postchallenge anti-N antibody response and, 2) PRNT
titers that remained essentially unchanged after SEOV
challenge.   Although the IFAT results indicated that
none of the pWRG/SEO-M vaccinated hamsters developed
20   anti-N antibodies after challenge, the more sensitive
anti-N ELISA revealed that three hamsters had a weak
anti-N antibody response.   Because the postchallenge
anti-N ELISA GMT of these hamsters (GMT = 50) was more
than 50 times lower than the negative control pWRG7077
25   vaccinated hamsters (GMT > 3,195), it is likely the
anti-N antibodies represent an antibody response to
either the input SEOV antigen (no replication), or to
a limited infection (e.g., one round of replication).
The PRNT data provided additional evidence that
30   vaccination with pWRG/SEO-M, but not pWRG/SEO-S,
protected hamsters from SEOV infection.   Whereas the
negative control pWRG7077-vaccinated hamsters' PRNT
titers increased from <20 to between 10,280 and 81,920
after challenge, the pWRG/SEO-M-vaccinated hamster
35   PRNT titers remained within twofold of prechallenge

levels. Moreover, the postchallenge PRNT titers of
the pWRG/SEO-M-vaccinated hamsters (GMT = 368)
remained well below the postchallenge PRNT titers of
the pWRG7077-vaccinated hamsters (GMT > 27,024).

5   Hamsters vaccinated with pWRG/SEO-S were infected with
SEOV after challenge. The pWRG/SEO-S-vaccinated
hamsters' postchallenge PRNT titers increased from <20
to between 2,560 and 20,480. Although the
postchallenge PRNT titers of the pWRG/SEO-S were high

10   (GMT = 5,885), it is noteworthy that these titers were
approximately fivefold lower than the negative control
(pWRG7077) postchallenge titers (GMT > 27,024).

The absence of a neutralizing antibody response
in mice and hamsters vaccinated with pWRG/SEO-S, with

15   or without complement, is consistent with published
data that monoclonal antibodies to G1 and G2, but not
N, have neutralizing activity (Dantas *et al.*, 1986,
Virology 151, 379-384; Arikawa *et al.*, 1989, *J. Gen.
Virol.* 70, 615-624; Schmaljohn *et al.*, 1990, J. Virol.

20   64, 3162-3170; Arikawa *et al.*, 1992, *Arch. Virol.* 70,
615-624), and that vaccination with vaccinia
recombinants expressing G1 and/or G2, but not N,
elicit a neutralizing response (Pensiero *et al.*, 1988,
J. Virol. 62, 696-702; Schmaljohn *et al.* 1992, *Vaccine*

25   10, 10-13; Xu *et al.*, 1992, *Am. J. Trop. Med. Hyg.* 47,
397-404; Chu *et al.*, 1995, supra).

Failure of an anti-N immune response to protect
against infection was unexpected because others found
that vaccination with recombinant N expressed in

30   insect cells, bacteria, or as chimeric HBV core
particles, protect against infection (Schmaljohn *et
al.*, 1990, supra; Yoshimatsu *et al.*, 1993, *Arch.
Virol.* 130, 365-376; Lundkvist *et al.*, 1996, *Virology*
216, 397-406; Ulrich *et al.*, 1998, *Vaccine* 16, 272-

35   280). Also, vaccination with recombinant vaccinia

viruses expressing the SEOV S segment partially
protected gerbils from SEOV infection (Xu *et al.* 1992,
supra). Failure of naked DNA vaccination with the S
segment to protect hamsters in our experiments may be
5   related to a quantitative rather than qualitative
deficiency. Although four of five hamsters vaccinated
with pWRG/SEO-S developed a detectable antibody
response by IFAT and ELISA, the titers were relatively
low (ELISA titers less than or equal to 1:1,600). The
10   reason for these low anti-N antibody titers in
hamsters, but not mice, remains unclear. Fault cannot
lie with the gene gun cartridges as the same lot was
used to vaccinate both hamsters and mice on the same
day; and the mouse seroconversion rate was 90%, with
15   an ELISA GMT of over 7,000. Low antibody titers may
reflect a poor overall immune response to vaccination
with pWRG/SEO-S in hamsters or, alternatively, it is
possible that epidermal administration of S DNA with
the gene gun results in a predominantly cell-mediated,
20   rather than humoral, response to N. Nevertheless, our
findings demonstrate that there was a detectable anti-
N immune response in four of five hamsters after naked
DNA gene gun vaccination with pWRG/SEO-S, and that
this response, whether humoral and/or cell-mediated,
25   was insufficient to protect against SEOV infection.
The ~fivefold difference in postchallenge PRNT titers
of pWRG/SEO-S versus pWRG7077 vaccinated hamsters
suggests pWRG/SEO-S vaccination may nonetheless limit
SEOV infection.
30   We found that SEOV neutralizing activity elicited
in mice and hamsters was reduced 4- to 32-fold if the
sera was heat inactivated (56°C, 30 min). This loss
in neutralizing activity could be completely reversed
if guinea pig complement was included in the PRNT.
35   These data are consistent with those of others who

reported that hantavirus neutralizing activity in sera
from humans, mice, and especially rats, was enhanced
by complement (Takenaka *et al.*, 1985, *Arch. Virol.* 84,
197-206; Asada *et al.*, 1989, *J. Gen. Virol.* 70, 819-

5   825). The observation that complement plays a major
role in hantavirus neutralization is notable because
PRNT titers are commonly used to assess the
immunogenicity and predicted efficacy of hantavirus
vaccines. Conditions that deplete or add complement,

10   such as heat inactivation or addition of exogenous
complement (e.g., as a purified protein fraction or as
fresh animal sera), could alter PRNT titers and,
therefore, could alter the assessment of candidate
vaccines.

15   Future studies are aimed at developing naked DNA
vaccines that protect against other HFRS-associated
hantaviruses, including HTNV, DOBV, and PUUV; and also
against the recently discovered North American
hantaviruses associated with hantavirus pulmonary

20   syndrome (reviewed in Schmaljohn and Hjelle, 1997,
Emerg. Infect. Dis. 3, 95-104). We are also
interested in evaluating the protective efficacy of
candidate vaccines against increasing challenge doses,
as well as different routes of challenge.

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